Electronic Journal of Biotechnology 18 (2015) 320–326

HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

# Analysis of a suppressive subtractive hybridization library of *Alternaria alternata* resistant to 2-propenyl isothiocyanate

Heriberto García-Coronado [a], Rosalba Troncoso-Rojas [b], Martín Ernesto Tiznado-Hernández [b], María del Carmen de la Cruz Otero [a], Sylvia Páz Díaz-Camacho [a], María Elena Báez-Flores [a,*]

[a] Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa, Ciudad Universitaria, Culiacán, Sinaloa, Mexico
[b] Coordinación de Tecnología de Alimentos de Orígen Vegetal, Centro de Investigación en Alimentación y Desarrollo AC, Hermosillo, Sonora, Mexico

ARTICLE INFO

*Article history:*
Received 3 February 2015
Accepted 6 May 2015
Available online 27 June 2015

*Keywords:*
Blast2GO analysis
Fungal drug tolerance
Isothiocyanates
Natural fungicides

ABSTRACT

*Background:* Isothiocyanates (ITCs) are natural products obtained from plants of the Brassicas family. They represent an environmentally friendly alternative for the control of phytopathogenic fungi. However, as it has been observed with synthetic fungicides, the possibility of inducing ITC-resistant strains is a major concern. It is, therefore, essential to understanding the molecular mechanisms of fungal resistance to ITCs. We analyzed a subtractive library containing 180 clones of an *Alternaria alternata* strain resistant to 2-propenyl ITC (2-pITC). After their sequencing, 141 expressed sequence tags (ESTs) were identified using the BlastX algorithm. The sequence assembly was carried out using CAP3 software; the functional annotation and metabolic pathways identification were performed using the Blast2GO program.
*Results:* The bioinformatics analysis revealed 124 reads with similarities to proteins involved in transcriptional control, defense and stress pathways, cell wall integrity maintenance, detoxification, organization and cytoskeleton destabilization; exocytosis, transport, DNA damage control, ribosome maintenance, and RNA processing. In addition, transcripts corresponding to enzymes as oxidoreductases, transferases, hydrolases, lyases, and ligases, were detected. Degradation pathways for styrene, aminobenzoate, and toluene were induced, as well as the biosynthesis of phenylpropanoid and several types of N-glycan.
*Conclusions:* The fungal response showed that natural compounds could induce tolerance/resistance mechanisms in organisms in the same manner as synthetic chemical products. The response of *A. alternata* to the toxicity of 2-pITC is a sophisticated phenomenon including the induction of signaling cascades targeting a broad set of cellular processes. Whole-transcriptome approaches are needed to elucidate completely the fungal response to 2-pITC.



## 1. Introduction

The use of synthetic fungicides in agriculture caused the development of drug-resistant fungal strains [1]. The presence of toxic residues in agricultural products may have potentially adverse effects on human health [2], the environment [3], and biodiversity [4,5]. It is, therefore, important to reduce the dependency on synthetic fungicides to control phytopathogenic fungi. For this purpose, natural fungicides of plant origin are being explored. Among them, isothiocyanates (ITCs) represent promising alternatives to synthetic fungicides for the control of fungi causing postharvest fruit losses [6,7]. In addition, ITCs have attracted attention in cancer research because of their ability to inhibit carcinogenesis and cancer growth in both *in vitro* and *in vivo* models [8].

ITCs are compounds synthesized by plants from the *Brassicaceae* family, such as radish, cauliflower, and mustard. They have a potent fungicidal activity against a number of fungi, including *Alternaria alternata* [6]. *A. alternata* is a fungus that causes spot lesions on the leaves [9] and fruits [10] of a broad variety of hosts [11,12], and is considered an important generalist phytopathogen in the field and during postharvest. Another *Alternaria* species, *Alternaria brassicicola* is a specialist, which is infective to the plants of the genus *Brassicas* [13]. Because *Brassicas* plants produce ITCs as a defense mechanism against infectious microorganisms or predators, *A. brassicicola* developed a particular resistance mechanism against this strong selective pressure during their coevolution [14]. Thus, *A. brassicicola* acquired special mechanisms to resist ITCs, but such mechanisms are not present in the generalist fungus *A. alternata*. Because *A. alternata* is phylogenetically close to *A. brassicicola*, we hypothesized that *A. alternata* would respond and survive to the toxic effects of ITCs. Thus, *A. alternata* may be a useful model to study the molecular mechanisms activated in response to ITCs.

* Corresponding author.
*E-mail address:* elenabf@uas.edu.mx (M.E. Báez-Flores).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.06.002

In the previous work [15], *A. alternata*, a fungus naturally sensitive to ITCs, was found to acquire tolerance or resistance to the ITCs toxicity after a constant and prolonged exposure. That work suggested that the prolonged use of natural fungicides could induce the emergence of resistant strains, as it has been observed with synthetic chemical products.

For the adaptation to fungicides, fungi use various mechanisms [16]. With regard to ITCs, Sellam and coworkers [17] reported that the response of *A. brassicicola* to 2p-ITC was similar to that observed under oxidative stress conditions since 35% of the transcriptionally induced genes corresponded to glutathione S-transferase (GST), glutathione peroxidase, glutamyl cysteine synthetases, thioredoxins, thioredoxin-reductases, oxidoreductases, and cytochrome P450. In addition, the mechanisms for reducing the intracellular accumulation of 2p-ITC were induced. In total, 16% of the induced genes were identified as encoding mainly ATP-binding cassettes (ABCs) and major facilitator superfamily transporter proteins [17]. In our previous work, we constructed and analyzed a suppressive subtracted hybridization (SSH) library from the mycelia of *A. alternata* treated with 2-pITC, and found expressed sequence tags (ESTs) coding RNA-binding domains and integral membrane proteins, such as ABC CDR4 transporters, opsins, ATPases, and fumarate reductases [18]. In addition, we detected the sequences coding modulating proteins of the calmodulin family (EF-hand $Ca^{++}$) and hypothetical S-nitrosoglutathione [18]. Although these results suggest possible molecular mechanisms of *A. alternata* adaptation to 2-pITC, additional adaptation mechanisms, including numerous metabolic pathways, exist because there are many more unexplored differentially expressed clones in the SSH library. In our previous work, a forward library was constructed, which allowed us to identify the genes that were being expressed in the treated organism but not in the control organism. Thus, the differentially expressed genes could be directly involved in the resistance process.

In this study, we analyzed 180 such unexplored clones from the forward SSH library constructed from *A. alternata* tolerant to 2-pITC. Because of their origin and nature, these unstudied clones represent a valuable genetic resource to provide additional scientific information on the molecular adaptation mechanisms of *A. alternata* to ITCs. Since the number of clones in this study was higher than in the first round of analysis, we expected to find different transcripts that were involved in previously unidentified adaptation processes in *A. alternata* 2-pITC tolerance, or were involved in known adaptation pathways to toxic compounds in other organisms. Indeed, we found a very diverse number of transcripts encoding for proteins or enzymes not detected in the first screening of the library. Further, they were not reported in previous studies focusing on the *Alternaria* tolerance to natural or synthetic compounds. These results are significant and complement previous works including ours because they reveal transcripts regulating the expression of genes and, allowed us to visualize genetic networks that are activating metabolic pathways to alleviate the toxic effect of 2-pITC on *A. alternata*.

## 2. Materials and methods

### 2.1. Library construction

A forward SSH library was constructed following the protocol of the provider company (Clontech, Palo Alto, CA, USA). The details regarding the *A. alternata*, SSH library construction can be reviewed in Baez-Flores et al. [18]. Briefly, the mRNA was isolated from *A. alternata* strain adapted to lethal levels of 2-propenyl-isothiocyanate according to the protocol published by Islas-Flores et al. [19]. cDNAs were prepared using the SMART PCR cDNA synthesis kit and subtracted with the PCR-Select DNA Subtraction Procedure (Clontech, Palo Alto CA). The differentially expressed cDNAs were cloned into p-GEM-T Easy vector and cells of *E. coli* JM109 were transformed with them (Promega, Madison, WI).

### 2.2. Plasmid DNA extraction and sequencing of differentially expressed ESTs

Clones harboring ESTs in the pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA), from the 2-pITC-treated *A. alternata* SSH library, were reactivated in LB agar. Then, the clones were cultivated in an LB-ampicillin broth for 24 h. Plasmid DNA was extracted using the alkaline lysis method [20] and digested with the *Rsa*I restriction enzyme (New England Biolabs® Inc. Ipswich, MA, USA). The restriction products were electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized in a transilluminator set at 312 nm (LA-20E; VWR Scientific, Buffalo Grove, IL, USA). The size of the insert in each clone was confirmed. The plasmid DNA was sent for sequencing (Genomic Analysis and Technology Core Facility, University of Arizona, AZ, USA) using the Sanger dideoxy sequencing technique and the M13 forward oligonucleotide.

### 2.3. Assembly and BLAST analysis of the sequences

To eliminate the DNA of vector origin and the adaptors used for the differentially expressed fragments' amplification, the obtained sequences were analyzed using the VecScreen program available at the National Center for Biotechnology Information (NCBI) webpage. Then, sequences corresponding to the same genes were assembled using the CAP3 Sequence Assembly Software [21]. The contigs and singletons generated were analyzed by the BLAST program using the algorithms BlastN [22] and BlastX [23].

### 2.4. Submission of genetic sequences to DNA databases

The sequences collected in this work were deposited in DDBJ/EMBL/GenBank using the Sequin software available at the NCBI web page. The ESTs were deposited under the accession numbers JK036089–JK036229. The contigs were deposited as Transcriptome Shotgun Assembly project at DDBJ/EMBL/GenBank under Bioproject PRJNA260095. The *A. alternata* Transcriptome Shotgun Assembly Project (TSA) has the accession number GBZG00000000. The version described in this paper is the first version (GBZG01000000) and consists of sequences GBZG01000001–GBZG01000030.

### 2.5. Sequence annotation

To assign biological functions to the transcripts encoded by the differentially expressed genes, a functional annotation of contigs and singletons was performed using the BLAST2GO (B2GO) software version 2.7.0 [24]. The analysis was carried out against the non-redundant nucleotide collection of GenBank with a minimum *E*-value of $1 \times 10^{-6}$ and a high-scoring segment pair cut-off of 33. The annotation step was carried out using the program default parameters and expanded using ANNEX (Annotation Expander). A B2GO InterPro Scan [25] was performed to search for additional GO terms corresponding to functional domains. The ESTs with GO annotations received enzyme codes (EC), and the B2GO KEGG module retrieved the maps of metabolic pathways in which the tracked EC numbers participated.

## 3. Results

### 3.1. Assembly and Blast analysis

Of 180 sequenced clones, 141 ESTs met the quality requirements after sequence cleaning. The CAP3 Assembly resulted in 124 reads, consisting of 40 contigs and 84 singletons. The Blast search returned 58% of the reads with similarities to characterized proteins, 25.8%

matched hypothetical proteins, and 12% had no similarity to known sequences. Some of the reads showing significant similarities with known proteins are listed in Table 1, whereas the ESTs contained in each resultant contig are listed in Table 2. Of the sequences with similarities to hypothetical proteins, 8% were similar to *Pyrenophora teres* f. terms, 5.6% to *Pyrenophora tritici-repentis*, and 4% to *Setosphaeria turcica* proteins.

Among the sequences similar to known proteins were those corresponding to regulatory checkpoint 1 (CHK1) and WSC domain-containing proteins, LemA and major Woronin body proteins, SLA1- and CFEM-domain GPI-anchored proteins, and GTP-binding, zinc finger and actin-bundling proteins. Also, we found ESTs similar to oxidoreductases enzymes (EC 1.2.1.23, EC 1.3.11.60, EC 1.3.8.8, and EC 1.2.1.65); transferases (EC 2.1.1, and EC 2.7.8); hydrolases

**Table 1**
Sequences from a suppressive subtracted hybridization library of *A. alternata* resistant to 2-propenyl isothiocyanate that are similar to known proteins. The results were obtained using the BlastX algorithm against the non-redundant database from GenBank (*E*-value < 1e−05).

| Sequence ID | GenBank accession number | Size (bp) | Similar sequence in GB/organism/accession number | Coverage % | E-value | Identity % |
|---|---|---|---|---|---|---|
| Aaitc271 | JK036093 | 573 | Similar to 60S ribosomal protein L28 [*Leptosphaeria maculans* JN3] > emb\|CBY00944.1\| | 47% | 2e−50 | 90% |
| Aaitc277 | JK036098 | 382 | Vacuolar ATP synthase catalytic subunit A [*Pyrenophora tritici-repentis* Pt-1C-BFP] > gb\|EDU43928.1\| | 91% | 1e−68 | 94% |
| Aaitc293 | JK036107 | 420 | Putative chitin- domain 3 protein [*Botryotinia fuckeliana* BcDW1] EMR83340.1 | 36% | 3e−08 | 56% |
| Aaitc295 | JK036108 | 699 | Woronin body major protein [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU41317.1\| | 54% | 2e−17 | 58% |
| Aaitc297 | JK036109 | 404 | Similar to hydrolase [*L. maculans* JN3] > emb\|CBX99475.1\| | 94% | 7e−71 | 87% |
| Aaitc300 | JK036112 | 448 | 40S ribosomal protein S26 [*Tuber melanosporum* Mel28] > emb\|CAZ81138.1\| | 33% | 8e−15 | 94% |
| Aaitc311 | JK036120 | 437 | ATP synthase subunit alpha, mitochondrial precursor [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU50467.1\| | 37% | 2e−21 | 82% |
| Aaitc314 | JK036123 | 379 | 40S ribosomal protein S17 [*Aspergillus fumigatus* Af293] | 69% | 3e−54 | 95% |
| Aaitc318 | JK036127 | 457 | Cystathionine gamma-lyase [*P. tritici-repentis* Pt-1C-BFP] | 38% | 2e−25 | 88% |
| Aaitc323 | JK036129 | 410 | Translational activator [*Colletotrichum orbiculare* MAFF 240422] ENH88059.1 | 48% | 4e−13 | 79% |
| Aaitc324 | JK036130 | 406 | GPI anchored CFEM domain containing protein [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU40367.1\| | 62% | 5e−42 | 83% |
| Aaitc326 | JK036131 | 571 | Cytoskeleton assembly control protein SLA1 [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU50968.1\| | 26% | 1e−23 | 96% |
| Aaitc332 | JK036134 | 732 | GTP-binding protein 128up [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU39679.1\| | 59% | 2e−96 | 97% |
| Aaitc344 | JK036142 | 575 | Similar to phosphatidyl synthase [*L. maculans* JN3] > emb\|CBX91093.1\| | 55% | 7e−54 | 81% |
| Aaitc346 | JK036144 | 456 | Zinc finger containing protein [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU49999.1\| | 59% | 1e−41 | 77% |
| Aaitc383 | JK036152 | 610 | 40S ribosomal protein S27 [*Pyrenophora teres* f. teres 0–1] > gb\|EFQ88323.1\| | 50% | 1e−56 | 92% |
| Aaitc388 | JK036155 | 516 | Putative actin-bundling protein [*Neofusicoccum parvum* UCRNP2] EOD46166.1 | 94% | 2e−95 | 89% |
| Aaitc405 | JK036165 | 388 | Glycoside hydrolase family 16 protein [*Setosphaeria turcica* Et28A] EOA83343.1 | 51% | 9e−21 | 89% |
| Aaitc411 | JK036169 | 423 | rRNA methyltransferase NOP1 [*Pyrenophora teres* f. teres 0–1] > gb\|EFQ87989.1\| | 73% | 2e−66 | 99% |
| Aaitc442 | JK036185 | 497 | Carbohydrate-binding module family 18 protein [*Bipolaris maydis* C5] > gb\|ENI09831.1\| | 85% | 2e−83 | 90% |
| Aaitc449 | JK036189 | 607 | Salicylaldehyde dehydrogenase [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU43682.1\| | 67% | 4e−66 | 86% |
| Aaitc456 | JK036193 | 348 | 40S ribosomal protein S26 [*T. melanosporum* Mel28] > emb\|CAZ81138.1\| | 30% | 3e−05 | 97% |
| Aaitc457 | JK036194 | 401 | Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase [*Pseudozyma hubeiensis* SY62] | 95% | 1e−32 | 64% |
| Aaitc462 | JK036198 | 490 | Similar to importin beta-4 subunit [*L. maculans* JN3] > emb\|CBX96195.1\| | 74% | 2e−68 | 93% |
| Aaitc464 | JK036200 | 308 | Arrestin domain containing protein [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU48753.1\| | 88% | 2e−51 | 91% |
| Aaitc466 | JK036201 | 400 | Mitochondrial fusion GTPase protein [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU42985.1\| | 95% | 1e−73 | 94% |
| Aaitc473 | JK036207 | 853 | Streptomycin biosynthesis protein StrI [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU51230.1\| | 93% | 7e−158 | 78% |
| Aaitc474 | JK036208 | 727 | Pentatricopeptide repeat protein [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU39902.1\| | 97% | 1e−141 | 90% |
| Aaitc485 | JK036216 | 323 | Histone H2A [*Coniosporium apollinis* CBS 100218] EON65154.1 | 31% | 3e−12 | 97% |
| Aaitc490 | JK036221 | 526 | ABC transporter CDR4 [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU44273.1\| | 97% | 4e−50 | 59% |
| Aaitc493 | JK036224 | 480 | Iron sulfur cluster assembly protein 1, mitochondrial precursor [*P. tritici-repentis* Pt-1C-BFP] > gb\|EDU44735.1\| | 71% | 1e−75 | 98% |

**Table 2**
ESTs in contigs.

| Contigs (Lab ID) | Contig accession number (TSA) | ESTs in contig (Lab ID) | EST GenBank accession number |
|---|---|---|---|
| Aaitcas18 | GBZG01000001 | Aaitc412 | JK036170 |
| | | Aaitc273 | JK036095 |
| | | Aaitc455 | JK036192 |
| Aaitcas19 | GBZG01000002 | Aaitc389 | JK036156 |
| | | Aaitc274 | JK036096 |
| | | Aaitc394 | JK036158 |
| | | Aaitc482 | JK036214 |
| Aaitcas20 | GBZG01000003 | Aaitc448 | JK036229 |
| | | Aaitc290 | JK036105 |
| | | Aaitc491 | JK036222 |
| Aaitcas21 | GBZG01000004 | Aaitc306 | JK036116 |
| | | Aaitc307 | JK036117 |
| | | Aaitc434 | JK036181 |
| | | Aaitc470 | JK036205 |
| Aaitcas22 | GBZG01000005 | Aaitc381 | JK036150 |
| | | Aaitc343 | JK036141 |
| | | Aaitc425 | JK036172 |
| | | Aaitc268 | JK036090 |
| | | Aaitc331 | JK036133 |
| | | Aaitc387 | JK036154 |
| | | Aaitc495 | JK036226 |
| | | Aaitc496 | JK036227 |
| | | Aaitc461 | JK036197 |
| | | Aaitc481 | JK036213 |
| Aaitcas23 | GBZG01000006 | Aaitc428 | JK036175 |
| | | Aaitc358 | JK036149 |
| | | Aaitc341 | JK036140 |
| | | Aaitc329 | JK036132 |
| | | Aaitc450 | JK036190 |
| | | Aaitc410 | JK036168 |
| | | Aaitc467 | JK036202 |
| Aaitcas24 | GBZG01000007 | Aaitc349 | JK036146 |
| | | Aaitc435 | JK036182 |
| | | Aaitc489 | JK036220 |
| Aaitcas25 | GBZG01000008 | Aaitc441 | JK036184 |
| | | Aaitc409 | JK036167 |
| | | Aaitc477 | JK036210 |
| Aaitcas26 | GBZG01000009 | Aaitc278 | JK036099 |
| | | Aaitc458 | JK036195 |
| Aaitcas27 | GBZG01000010 | Aaitc356 | JK036148 |
| | | Aaitc484 | JK036215 |
| Aaitcas28 | GBZG01000011 | Aaitc399 | JK036160 |
| | | Aaitc492 | JK036223 |
| Aaitcas29 | GBZG01000012 | Aaitc475 | JK036209 |
| | | Aaitc401 | JK036161 |
| | | Aaitc288 | JK036103 |
| | | Aaitc267 | JK036089 |
| | | Aaitc285 | JK036102 |
| | | Aaitc404 | JK036164 |
| | | Aaitc403 | JK036163 |
| | | Aaitc460 | JK036196 |
| | | Aaitc471 | JK036206 |
| Aaitcas30 | GBZG01000013 | Aaitc478 | JK036211 |
| | | Aaitc426 | JK036173 |
| Aaitcas31 | GBZG01000014 | Aaitc486 | JK036217 |
| | | Aaitc494 | JK036225 |
| Aaitcas32 | GBZG01000015 | Aaitc488 | JK036219 |
| | | Aaitc469 | JK036204 |
| Aaitcas33 | GBZG01000016 | Aaitc338 | JK036138 |
| | | Aaitc1A | |
| Aaitcas34 | GBZG01000017 | Aaitc348 | JK036145 |
| | | Aaitc33 | |
| Aaitcas35 | GBZG01000018 | Aaitc427 | JK036174 |
| | | Aaitc65 | |
| Aaitcas36 | GBZG01000019 | Aaitc430 | JK036177 |
| | | Aaitc161 | |
| Aaitcas37 | GBZG01000020 | Aaitc463 | JK036199 |
| | | Aaitc133 | |
| Aaitcas38 | GBZG01000021 | Aaitc143 | |
| | | Aaitc46 | |
| Aaitcas39 | GBZG01000022 | Aaitc150 | |
| | | Aaitc436 | JK036183 |
| | | Aaitc301 | JK036113 |
| Aaitcas40 | GBZG01000023 | Aaitc227 | |
| | | Aaitc315 | JK036124 |
| Aaitcas41 | GBZG01000024 | Aaitc289 | JK036104 |
| | | Aaitc276 | JK036097 |
| Aaitcas42 | GBZG01000025 | Aaitc299 | JK036111 |
| | | Aaitc279 | JK036100 |
| Aaitcas43 | GBZG01000026 | Aaitc322 | JK036128 |
| | | Aaitc305 | JK036115 |
| Aaitcas44 | GBZG01000027 | Aaitc345 | JK036143 |
| | | Aaitc316 | JK036125 |
| Aaitcas45 | GBZG01000028 | Aaitc335 | JK036136 |
| | | Aaitc269 | JK036091 |
| | | Aaitc304 | JK036114 |
| | | Aaitc308 | JK036118 |
| | | Aaitc310 | JK036119 |
| | | Aaitc317 | JK036126 |
| Aaitcas46 | GBZG01000029 | Aaitc443 | JK036186 |
| | | Aaitc337 | JK036137 |
| | | Aaitc429 | JK036176 |
| Aaitcas47 | GBZG01000030 | Aaitc447 | JK036188 |
| | | Aaitc451 | JK036191 |
| | | Aaitc433 | JK036180 |

(EC 3.6.5.1, EC 3.2.1, EC 3.1.1.2, EC 3.6.4.13, and EC 3.6.3.14); a lyase (EC 4.4.1.1); and a ligase (EC 6.2.1.17).

### 3.2. Functional annotation and metabolic pathways

In the functional annotation procedure, the B2GO software assigned GO terms to 54% of the reads set, whereas no results were obtained for 13%, 22%, and 9.6% of the sequences during the blasting, mapping, or annotation processes, respectively. A total of 265 annotations and 17 EC numbers (for 16 sequences) were retrieved. The annotation distribution, according to GO, showed that the best-represented categories at level two were molecular function and biological process. In the molecular function category (Fig. 1a), more transcripts were assigned to binding (38) and catalytic activity (33) followed by structural molecule activity (13). In the biological process category (Fig. 1b), the main clusters were cellular and metabolic process (both with 44 GO terms) followed by single organisms and localization (22 and 17 GO terms, respectively).

Based on the EC codes assigned to sequences and the KEGG maps retrieved by B2GO, we obtained transcripts having enzymatic functions involved in the metabolic pathways. The exposure of *A. alternata* to 2-pITC, differentially induced enzymes participating in the metabolism of nitrogen, pyruvate, cysteine and methionine, selenium compounds, propanoate, phenylalanine, butanoate, methane, glycine, serine, and threonine. Also, the enzymes participating in styrene, aminobenzoate, and toluene degradation were induced. Furthermore, the enzymes involved in catalyzing steps in glycolysis, gluconeogenesis, citrate cycle, oxidative phosphorylation, and carbon fixation pathways, were detected. Finally, the enzymes involved in the phenylpropanoid pathway and several types of N-glycan biosynthesis were also differentially induced.

## 4. Discussion

Among the differentially expressed ESTs of *A. alternata* tolerant to 2-pITC, Aaitc346 (GenBank accession number JK036144) showed significant similarity to a Zn-finger transcription factor. This protein is induced, along with ABC transporters, in *Aspergillus fumigatus* after exposure to voriconazole [26] and in *Fusarium graminearum* after exposure to tebuconazole [27]. In plants, these proteins are involved in defense pathways and are induced in response to several types of stress [28]. In *F. graminearum*, the Zn-finger transcription factor tac1p, which is a transcriptional activator of *CDR* genes, regulates the expression of *CDR1* and *CDR2*, which encode ABC transporters in

azole-resistant clinical isolates of *Candida albicans* [29]. Moreover, *Botrytis cinerea* that are resistant to several fungicides overexpress an ABC transporter, which is induced by a mutation in the putative Zn-finger transcription factor Mrr1 [30]. In our previous work, using real-time RT-PCR, we confirmed the overexpression of an ABC transporter in *A. alternata* in response to 2-pITC [18]. Thus, the Zn-finger transcription factor is induced by 2-pITC, which activates the expression of the ABC multidrug CDR4 transporters in *A. alternata* to provide tolerance to 2p-ITC.

In our current work, we found a sequence similar to a protein containing a WSC domain [31]. The WSC1 gene encodes an integral membrane protein (Wsc1p) that functions as a stress sensor. In yeast, this protein participates in the monitoring of cell wall integrity and the activation of the protein kinase C (PKC) pathway in response to external stress signals, such as cell wall perturbations. Wscp1 also regulates 1,3-β-glycan synthesis [31,32], a metabolic pathway identified in the B2GO analysis.

Among the 2p-ITC induced-transcripts of *A. alternata*, we also found a sequence encoding a karyopherin, Kap123. This molecule has an important role in cell integrity, which also depends on PKC pathway activity that regulates the secretion and vesicular transport pathway. The cellular integrity pathway is activated after cell wall damage [33]. Other sequences involved in cellular integrity are those encoding the major Woronin body protein, the GPI-anchored CFEM domain-containing protein, and the oxidoreductase glyoxal oxidase. The Woronin bodies are proteinic corpuscles that move to the septal pore of filamentous fungi in response to cellular damage [34], whereas the GPI-anchored CFEM domain-containing proteins are involved in cell wall stabilization [35]. Glyoxal oxidase may have a role in fungal detoxification [36]. The induction of this enzyme in 2p-ITC-treated *A. alternata* suggests it has a role in the detoxification of 2p-ITC.

Other genes induced in *A. alternata* after 2p-ITC treatments are GTPases. Some of these molecules are activated by stress, and in turn, they activate the Pkc1 kinase in the PKC pathway [32]. The interruption of CgBem2, which encodes a GTPases activator protein in *Candida glabrata*, resulted in azole susceptibility, suggesting a function for GTPases in the survival to stress caused by antimycotics [37]. Thus, the induction of GTPases in *A. alternata* after 2p-ITC treatments suggests its participation in the resistance to 2p-ITC.

In our library, we also found a sequence similar to an SLA1 protein, an actin-cytoskeleton regulatory complex component (PAN1), and an ubiquitin-binding protein [38]. Moreover, actin-binding proteins that direct the response to external stimuli [39] were detected. Additionally, sequences similar to proteins containing Lem domains were identified. In yeast, these proteins participate in the genome and nuclear structure organization [40] while, in *Caenorhabditis elegans*, they have a role in the response to DNA damage [41]. Another transcript induced by 2p-ITC codes for the regulatory protein CHK1, which is also involved in responses to DNA damage and cell survival [42]. In *Clonostachys rosea*, the CHK1 protein was induced in response to oxidative stress by toxins [43]. Because 2p-ITC is a compound causing oxidative stress, it is feasible that CHK1 was induced in *A. alternata* after the 2p-ITC treatment.

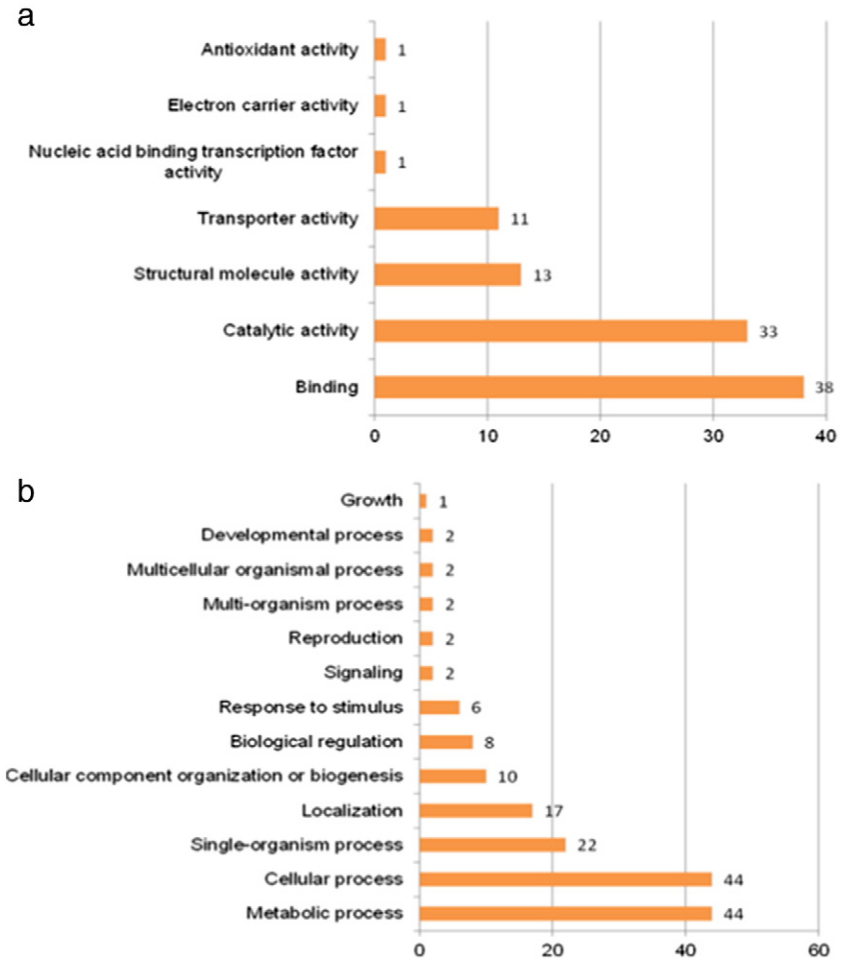


**Fig. 1.** Results of the annotation of sequences from a suppressive subtracted hybridization library of *A. alternata* resistant to 2-propenyl isothiocyanate. The distribution of gene ontology (GO) terms is shown at a GO level 2. The number in each category shows the frequency for each GO term. a) Molecular function; b) Biological process.

Several sequences detected in the library suggest that 2p-ITC induces a large variety of proteins and enzymes involved in the activation of signal cascades that promote general cellular responses oriented to cell reparation and maintenance. Such sequences encode 40S (S0, S17, S18, S26, and S27) and 60S (P0, L20, L21, and L28) ribosomal subunits; 1-α-elongation factor; methyltransferases; transcripts involved in amino acid biosynthesis (aminotransferase and cystathionine gamma-lyase); sequences participating in fungal pH adaptation (arrestin domain-containing proteins) [44]; proteins implicated in RNA metabolism (pentatricopeptide repeat motif) [45] or, functioning as chaperones (DEAD box proteins) [46]. Additionally, were identified the transcripts involved in the regulation of genetic expression (Fe–S clusters) [47] and cell cycle progression, as well as in ribosome biogenesis (GTP-binding proteins) [48]. According to their reported functions, none of these proteins can directly confer resistance to the fungus against fungicides. However, the overexpression of these molecules provides protection against the effects of 2p-ITC on the structure and function of the fungal cell. Thus, the cell uses all possible genetic, biochemical, and structural strategies to survive the toxicity of 2p-ITC.

In our previous study, the tolerance of *A. alternata* to 2p-ITC required mainly calcium ions and the efflux of the compound by an ABC transporter. There is evidence that the genes induced in *A. alternata* by 2p-ITC, are also expressed in response to synthetic fungicides [26]. When *A. alternata* was treated with the synthetic compound carbonyl sulfide, 510 cDNAs differentially expressed were found. These genes are related to general metabolism, growth, cellular division, defense, cellular transport, and signal transduction [49], similar to our work results. Therefore, it should be emphasized that even natural compounds could induce tolerance/resistance mechanisms in organisms in the same manner as synthetic chemical products if they were not used properly. In a study with fungal toxins Kosawang et al. [43] using a SSH experimental approach reported 443 and 446 differentially expressed clones induced in *Clonostachys rosea* by deoxynivalenol (DON) and zearalenone (ZEA) toxins, respectively. DON induced proteins involved in the stress response as well as metabolic enzymes (cytochromes c oxidase and P450) while ZEA induced the detoxifying enzyme HD101 and ABC pleiotropic drug transporters. These authors concluded that the tolerance to fungal toxins in *C. rosea* was provided by a broad range of genes playing a role in metabolism and transport. Based on the increase of transcripts encoding the metabolic enzymes CYP450 and COX, the sugar transporters HXT2 and H+-ATPase, as well as the Hsp70 and Hsp90 proteins, they stated that the cellular energy was used to synthesize proteins that were inactivated by the toxins.

Several publications using a differential expression approach to studying the response to natural and synthetic compounds have reported different numbers of genes induced. However, the reported number of metabolic pathways by using more robust protocols is modest, considering the robustness of the techniques used. The number of genes reported as induced by synthetic compounds in the literature is higher than in this work. This fact is not related to the treatment, but rather to the methodology. For instance, using microarrays, Ferreira et al. [26] found 2271 genes differentially expressed in *A. fumigatus* exposed to voriconazole. These authors reported increased transcripts levels of genes involved in a variety of cellular functions: *e.g.* transporters, transcription factors as well as proteins involved in cell metabolism. In agreement with our results, these authors found that the induction of two C2H2 zinc finger domains, ATPase and calmodulin transcription factors putatively involved in the response to stress conditions imposed by the voriconazole treatment. These authors hypothesized that these proteins could have as target genes participating in detoxification (transporters).

Another study using microarrays in *F. graminearum* (Becher et al. [27]), reported 1058 differentially expressed genes in response to azole treatment and of them, 596 showed significantly increased transcript levels. The functional annotation of these genes found the ergosterol biosynthesis as an important pathway in the fungal response to azole. Also, the authors found transcripts differentially expressed encoding ABC transporters and transcription factors, presumably involved in mechanisms to decrease the toxic effect of the fungicide. They studied the expression level of 31 genes, out of which, they found five genes with increased transcript levels. These genes were involved in sterol metabolic processes. In contrast to our results, these authors reported a decreased expression of genes related to amino acid transport and metabolism. Even though this article reports a rather large study, the analysis of the genes with increased transcripts levels (GO annotation) identified only a few functional categories significantly represented; among them, the sterol biosynthetic process and tetracyclic and pentacyclic triterpene metabolism were found as the most represented functional categories. Additionally, isoprenoid metabolism and heme-binding proteins were important processes and functional category, respectively. The mentioned work found the induction of proteins with hexosyltransferase activity as well as proteins involved in membrane processes, mycelium development, respiratory chain, and energy generation. These authors assume that many of the induced genes exhibit a non-specific stress response caused by the azole membrane perturbations. However, they observed that the majority of the overexpressing genes responded specifically to the fungicide treatment. All of the pathways reported in that work were identified in our study. As compared with the mentioned published works, this is rather small due to the experimental approach utilized. However, we have a great variety of transcripts induced.

## 5. Concluding remarks

The results herein presented revealed a broader set of transcripts and cellular activities putatively implicated in the survival of *A. alternata* exposed to 2p-ITC. The induction of signaling cascades targeting diverse cellular processes is evident. These involve mainly the pathways of defense and stress response, cell wall integrity, cytoskeleton organization, and destabilization, as well as exocytosis and transport. Additionally, genome and nuclear structure organization, protein and ribosome synthesis, cell cycle progression, and DNA damage response activities were induced. Furthermore, some metabolic pathways underlying the fungal genetic responses against toxic compounds were identified. Interestingly, the proteins and enzymes that were induced in response to 2p-ITC can also be induced by the fungal resistance to different synthetic compounds. By performing a complete analysis of the *A. alternata* SSH library, a larger number of biological processes and molecular functions induced by 2p-ITC were identified. These findings show that the response of *A. alternata* to the toxic effects of 2-PITC is a complex and sophisticated defense mechanism. However, it is possible that the expression of some genes is elicited by a primary response of other genes to the toxicity of 2p-ITC, as suggested by the expression of genes related to the promotion of general cellular responses linked to growth and maintenance processes. To better understand the role for each transcript, RNA-seq and microarray studies should be performed. Thus, the complete knowledge of the tolerance mechanisms of *A. alternata* to 2-pITC will require the use of whole transcriptomic sequencing, genetic-level expression analyses, and functional analyses, which will allow us to study all of the genes involved and their functions in the resistance process.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Financial support

This study was funded by the Promotion and Support Program for Research Projects from the Universidad Autónoma de Sinaloa (Grant PROFAPI2010/012).

## Acknowledgments

The authors want to thank Dr. Yukifumi Nawa for his critical reading and contributions to the manuscript.

## References

[1] Mernke D, Dahm S, Walker AS, Laleve A, Fillinger S, Leroch M, et al. Two promoter rearrangements in a drug efflux transporter gene are responsible for the appearance and spread of multidrug resistance phenotype MDR2 in *Botrytis cinerea* isolates in French and German vineyards. Phytopathology 2011;101:1176–83. http://dx.doi.org/10.1094/PHYTO-02-11-0046.

[2] Alavanja MC, Bonner MR. Occupational pesticide exposures and cancer risk: A review. J Toxicol Environ Health Part B 2012;15:238–63. http://dx.doi.org/10.1080/10937404.2012.632358.

[3] Casida JE. The greening of pesticide-environment interactions: Some personal observations. Environ Health Perspect 2012;120:487–93. http://dx.doi.org/10.1289/ehp.1104405.

[4] Geiger F, Bengtsson J, Berendse F, Weisser WW, Emmerson M, Morales MB, et al. Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. Basic Appl Ecol 2010;11:97–105. http://dx.doi.org/10.1016/j.baae.2009.12.001.

[5] Jordaan MS, Reinecke SA, Reinecke AJ. Acute and sublethal effects of sequential exposure to the pesticide azinphos-methyl on juvenile earthworms (*Eisenia andrei*). Ecotoxicology 2012;21:649–61. http://dx.doi.org/10.1007/s10646-011-0821-z.

[6] Troncoso-Rojas R, Sánchez-Estrada A, Ruelas C, García HS, Tiznado-Hernández M. Effect of benzyl isothiocyanate on tomato fruit infection development by *Alternaria alternata*. J Sci Food Agric 2005;85:1427–34. http://dx.doi.org/10.1002/jsfa.2129.

[7] Tiznado-Hernández ME, Troncoso-Rojas R. Control of fungal diseases with isothiocyanates. Stewart Posthar Rev 2006;2:1–14. http://dx.doi.org/10.2212/spr.2006.1.4.

[8] Zhang Y. The molecular basis that unifies the metabolism, cellular uptake and chemopreventive activities of dietary isothiocyanates. Carcinogenesis 2012;33:2–9. http://dx.doi.org/10.1093/carcin/bgr255.

[9] Adachi Y, Tsuge T. Coinfection by different isolates of *Alternaria alternata* in single black spot lesions of Japanese pear leaves. Phytopathology 1994;84:447–51.

[10] Masunaka A, Ohtani K, Peever TL, Timmer LW, Tsuge T, Yamamoto M, et al. An isolate of *Alternaria alternata* that is pathogenic to both tangerines and rough lemon and produces two host-selective toxins, ACT- and ACR-Toxins. Phytopathology 2005;95:241–7. http://dx.doi.org/10.1094/PHYTO-95-0241.

[11] Thomma BPHJ. *Alternaria* spp.: From general saprophyte to specific parasite. Mol Plant Pathol 2003;4:225–36. http://dx.doi.org/10.1046/j.1364-3703.2003.00173.x.

[12] Troncoso-Rojas R, Tiznado-Hernández ME. *Alternaria alternata* (black rot, black spot). Postharvest decay: Control strategies; 2014 147–87. http://dx.doi.org/10.1016/B978-0-12-411552-1.00005-3.

[13] Rowe HC, Kliebenstein DJ. All mold is not alike: The importance of intraspecific diversity in necrotrophic plant pathogens. PLoS Pathog 2010;6:e1000759. http://dx.doi.org/10.1371/journal.ppat.1000759.

[14] Fournier E, Giraud T. Sympatric genetic differentiation of a generalist pathogenic fungus, *Botrytis cinerea*, on two different host plants, grapevine and bramble. J Evol Biol 2008;21:122–32. http://dx.doi.org/10.1111/j.1420-9101.2007.01462.x.

[15] Troncoso-Rojas R, Báez-Flores ME, Pryor B, García HS, Tiznado-Hernández ME. Inter simple sequence repeat polymorphism in *Alternaria* genomic DNA exposed to lethal concentrations of isothiocyanates. Afr J Microbiol Res 2013;7:838–52.

[16] Báez-Flores M-E, Troncoso-Rojas R, Tiznado-Hernandez M-E. Biochemical and genetic responses of fungi to the toxic effect of synthetic and natural fungicides. Am J Agric Biol Sci 2008;3:348–57. http://dx.doi.org/10.3844/ajabssp.2008.348.357.

[17] Sellam A, Dongo A, Guillemette T, Hudhomme P, Simoneau P. Transcriptional responses to exposure to the brassicaceous defence metabolites camalexin and allyl-isothiocyanate in the necrotrophic fungus *Alternaria brassicicola*. Mol Plant Pathol 2007;8:195–208. http://dx.doi.org/10.1111/j.1364-3703.2007.00387.x.

[18] Baez-Flores ME, Troncoso-Rojas R, Osuna MAI, Dominguez MR, Pryor B, Tiznado-Hernández ME. Differentially expressed cDNAs in *Alternaria alternata* treated with 2-propenyl isothiocyanate. Microbiol Res 2011;166:566–77. http://dx.doi.org/10.1016/j.micres.2010.11.004.

[19] Islas-Flores I, Peraza-Echeverría L, Canto-Canché B, Rodríguez-García CM. Extraction of high-quality, melanin-free RNA from *Mycosphaerella fijiensis* for cDNA preparation. Mol Biotechnol 2006;34:45–50. http://dx.doi.org/10.1385/MB:34:1:45.

[20] Green M, Sambrook J. Molecular cloning: A laboratory manual. 4th ed. New York: Cold Spring Harbor Laboratory Press; 2012.

[21] Huang X, Madan A. CAP3: A DNA sequence assembly program. Genome Res 1999;9: 868–77. http://dx.doi.org/10.1101/gr.9.9.868.

[22] Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. J Comput Biol 2000;7:203–14. http://dx.doi.org/10.1089/10665270050081478.

[23] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402. http://dx.doi.org/10.1093/nar/25.17.3389.

[24] Gotz S, Garcia-Gomez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, et al. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res 2008;36:3420–35. http://dx.doi.org/10.1093/nar/gkn176.

[25] Zdobnov EM, Apweiler R. InterProScan—An integration platform for the signature-recognition methods in InterPro. Bioinformatics 2001;17:847–8. http://dx.doi.org/10.1093/bioinformatics/17.9.847.

[26] Ferreira MES, Malavazi I, Savoldi M, Brakhage AA, Goldman MHS, Kim HS, et al. Transcriptome analysis of *Aspergillus fumigatus* exposed to voriconazole. Curr Genet 2006;50:32–44. http://dx.doi.org/10.1007/s00294-006-0073-2.

[27] Becher R, Weihmann F, Deising HB, Wirsel SGR. Development of a novel multiplex DNA microarray for *Fusarium graminearum* and analysis of azole fungicide responses. BMC Genomics 2011;12:52. http://dx.doi.org/10.1186/1471-2164-12-52.

[28] Gourcilleau D, Lenne C, Armenise C, Moulia B, Julien JL, Bronner G, et al. Phylogenetic study of plant Q-type C2H2 zinc finger proteins and expression analysis of poplar genes in response to osmotic, cold and mechanical stresses. DNA Res 2011;18: 77–92. http://dx.doi.org/10.1093/dnares/dsr001.

[29] Coste A, Selmecki A, Forche A, Diogo D, Bougnoux ME, d'Enfert C, et al. Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. Eukaryot Cell 2007;6:1889–904. http://dx.doi.org/10.1128/EC.00151-07.

[30] Kretschmer M, Leroch M, Mosbach A, Walker AS, Fillinger S, Mernke D, et al. Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. PLoS Pathog 2009;5: e1000696. http://dx.doi.org/10.1371/journal.ppat.1000696.

[31] Verna J, Lodder A, Lee K, Vagts A, Ballester R. A family of genes required for maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 1997;94:13804–9. http://dx.doi.org/10.1073/pnas.94.25.13804.

[32] Fernandes H, Roumanie O, Claret S, Gatti X, Thoraval D, Doignon F, et al. The Rho3 and Rho4 small GTPases interact functionally with Wsc1p, a cell surface sensor of the protein kinase C cell-integrity pathway in *Saccharomyces cerevisiae*. Microbiology 2006;152:695–708. http://dx.doi.org/10.1099/mic.0.28231-0.

[33] Reinoso-Martín C, Schüller C, Schuetzer-Muehlbauer M, Kuchler K. The yeast protein kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through activation of Slt2p mitogen-activated protein kinase signaling. Eukaryot Cell 2003;2:1200–10. http://dx.doi.org/10.1128/EC.2.6.1200-1210.2003.

[34] Beck J, Ebel F. Characterization of the major Woronin body protein HexA of the human pathogenic mold *Aspergillus fumigatus*. Int J Med Microbiol 2013;303:90–7. http://dx.doi.org/10.1016/j.ijmm.2012.11.005.

[35] Vaknin Y, Shadkchan Y, Levdansky E, Morozov M, Romano J, Osherov N. The three *Aspergillus fumigatus* CFEM-domain GPI-anchored proteins (CfmA-C) affect cell-wall stability but do not play a role in fungal virulence. Fungal Genet Biol 2014;63:55–64. http://dx.doi.org/10.1016/j.fgb.2013.12.005.

[36] Leuthner B, Aichinger C, Oehmen E, Koopmann E, Muller O, Muller P, et al. A $H_2O_2$-producing glyoxal oxidase is required for filamentous growth and pathogenicity in *Ustilago maydis*. Mol Genet Genomics 2005;272:639–50. http://dx.doi.org/10.1007/s00438-004-1085-6.

[37] Borah S, Shivarathri R, Kaur R. The Rho1 GTPase-activating protein CgBem2 is required for survival of azole stress in *Candida glabrata*. J Biol Chem 2011;286: 34311–24. http://dx.doi.org/10.1074/jbc.M111.264671.

[38] Uniprot. Actin cytoskeleton complex protein *SLA1*; 2014.

[39] Chang YC, Lamichhane AK, Kwon-Chung KJ. Role of actin-bundling protein Sac6 in growth of *Cryptococcus neoformans* at low oxygen concentration. Eukaryot Cell 2012;11:943–51. http://dx.doi.org/10.1128/EC.00120-12.

[40] Berk JM, Tifft KE, Wilson KL. The nuclear envelope LEM-domain protein emerin. Nucleus 2013;4:298–314. http://dx.doi.org/10.4161/nucl.25751.

[41] Dittrich CM, Kratz K, Sendoel A, Gruenbaum Y, Jiricny J, Hengartner MO. LEM-3 — A LEM domain containing nuclease involved in the DNA damage response in *C. elegans*. PLoS ONE 2012;7:e24555. http://dx.doi.org/10.1371/journal.pone.0024555.

[42] Tang J, Erikson RL, Liu X. Checkpoint kinase 1 (Chk1) is required for mitotic progression through negative regulation of polo-like kinase 1 (Plk1). Proc Natl Acad Sci U S A 2006;103:11964–9. http://dx.doi.org/10.1073/pnas.0604987103.

[43] Kosawang C, Karlsson M, Jensen DF, Dilokpimol A, Collinge DB. Transcriptomic profiling to identify genes involved in *Fusarium* mycotoxin Deoxynivalenol and Zearalenone tolerance in the mycoparasitic fungus *Clonostachys rosea*. BMC Genomics 2014;15:55. http://dx.doi.org/10.1186/1471-2164-15-55.

[44] Hervás-Aguilar A, Galindo A, Penalva MA. Receptor-independent ambient pH signaling by ubiquitin attachment to fungal arrestin-like PalF. J Biol Chem 2010; 285:18095–102. http://dx.doi.org/10.1074/jbc.M110.114371.

[45] Kuhl I, Dujeancourt L, Gaisne M, Herbert CJ, Bonnefoy N. A genome wide study in fission yeast reveals nine PPR proteins that regulate mitochondrial gene expression. Nucleic Acids Res 2011;39:8029–41. http://dx.doi.org/10.1093/nar/gkr511.

[46] Russell R, Jarmoskaite I, Lambowitz AM. Toward a molecular understanding of RNA remodeling by DEAD-box proteins. RNA Biol 2013;10:44–55. http://dx.doi.org/10.4161/rna.22210.

[47] Lill R, Muhlenhoff U. Iron–sulfur–protein biogenesis in eukaryotes. Trends Biochem Sci 2005;30:133–41. http://dx.doi.org/10.1016/j.tibs.2005.01.006.

[48] Bustelo XR, Sauzeau V, Berenjeno IM. GTP-binding proteins of the Rho/Rac family: Regulation, effectors and functions in vivo. Bioessays 2007;29:356–70. http://dx.doi.org/10.1002/bies.20558.

[49] Liu T, Li L, Wang Y, Zhan G, Liu B. Differential expression profiles of *Alternaria alternata* genes in response to carbonyl sulfide fumigation. J Microbiol 2010;48: 480–5. http://dx.doi.org/10.1007/s12275-010-9301-z.